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14. ABSTRACT We have demonstrated that the Triptolide (TPL), a diterpenoid tripoxides-like molecule (MW 360) purified from the herb *Tripterygium wilfordii Hook F* possesses a potent anti-tumor effect. To further enhance its targeting effect on tumor, TPL was conjugated with human RGD and NGR that highly expressed on vessel and tumor cells, and then further precessed in a liposome form as nanoparticle of RGD/NGR-PA-TPL-Liposome. The results of in vitro test RGD/NGR-PA-TPL-lipo showed a similar effect as TPL along. In the tumor bearing mice model, the tail vein injection showed a good targeting effect on vessel, however, after 6 injections of RGD/NGR-PA-TPL-Lipo all the treated mice died of allergy (fast breathing with a pattern of IgE type immune reaction) when 7 injection was carried out, indicating that the RGD/NGR is a foreign antigen to mouse. Then, we decided to conjugate TPL to hyaluronan (HA), the ligand to CD44 that is highly expressed on surface of breast cancer cells. The results of in vitro and in vivo tests showed that the new nanoparticle had a similar anti-tumor effect as TPL. Conclusion:1) in technique, RGD/NGR-PA-TPL-Lipo can be obtaines; 2) human RGD/NGR enhances the targeting effect on vessel; 3) RGD/NGR acts as a foreign antigen to mouse, perhaps to human too, therefore, it is not a good carrier to anti-tumor toxic agent; and 4) TPL itself is potent enough to kill cancer cells without further modification.

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Introduction

Most of anti-tumor drugs are derived from active molecules isolated from plants or micro-organisms, such as Taxol comes from Pacific yew (*T. brevifolia*). Among them, we are interested in Triptolide (TPL).

Triptolide (TPL) is a diterpenoid triepoxides-like molecule ($C_{20}H_{24}O_6$) with a molecular weight of 360.4 Dalton (1-4). It is purified from the herb *Tripterygium wilfordii Hook F* that has been used in China as a natural medicine for hundreds of years. TPL can inhibit actively proliferating cells, and thereby exerts anti-tumor activities (5-10). While the action mechanism of TPL is largely unknown, a few studies have suggested that it may act via induction of apoptosis (11-12). We have found that TPL could greatly reduce the *c-myc* and two pairs of major cell progression complexes, cyclin A/cdk2 and cyclin B/cdc2, and on the other hand, TPL could up-regulate apoptosis by activation of caspase 3 and PARP. Our special funding is that TPL also reduces the activity of telomerase, an idea target for cancer therapy.

The clinical trials of TPL as anti-tumor agent have been carried out in China. As shown in **Table 1,** the results were remarkable. When the therapeutic effect of TPL was evaluated in 21 patients, 10 of these patients experienced complete remission (CR, 47.6%); while 5 underwent a partially remission (PR). The total effective rate was 71.4%. There were different rates of remission in different types of acute leukemia. The highest rate of complete remission was achieved with acute granulocytic leukemia (75%), which was better than any current chemotherapy regimens for this disease.

Table 1. Effect of TPL on Different Types of Acute Leukemia

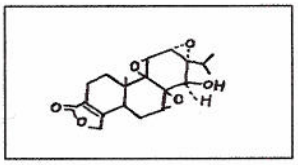
Types of leukemia	**Cases**	**CR**	**%**	**PR**	**%**	**NR**	**%**	**TR**	**%**
Granulocytic	8	6	**75.0**	1	12.5	1	12.5	7	**87.5**
Mononuclocytic	7	3	42.9	2	28.6	2	28.6	5	**71.5**
Lymphocytic	3			1	33.3	2	66.6	1	**33.3**
Erythroleukemic	2	1	50.0			1	50.0	1	**50.0**
Megakaryocytic	1			1	100.0			1	**100.0**
Total	**21**	**10**	**47.6**	**5**	**23.8**	**6**	**28.6**	**15**	**71.4**

This clinical data strongly suggest that TPL have a great potential of being developed as a novel anti-tumor agent.

In this study, we proposed to determine the effect of TPL on primary and metastatic **breast cancer** and to enhance the targeting effect of TPL on beast cancer using TPL nanoparticles.

Body

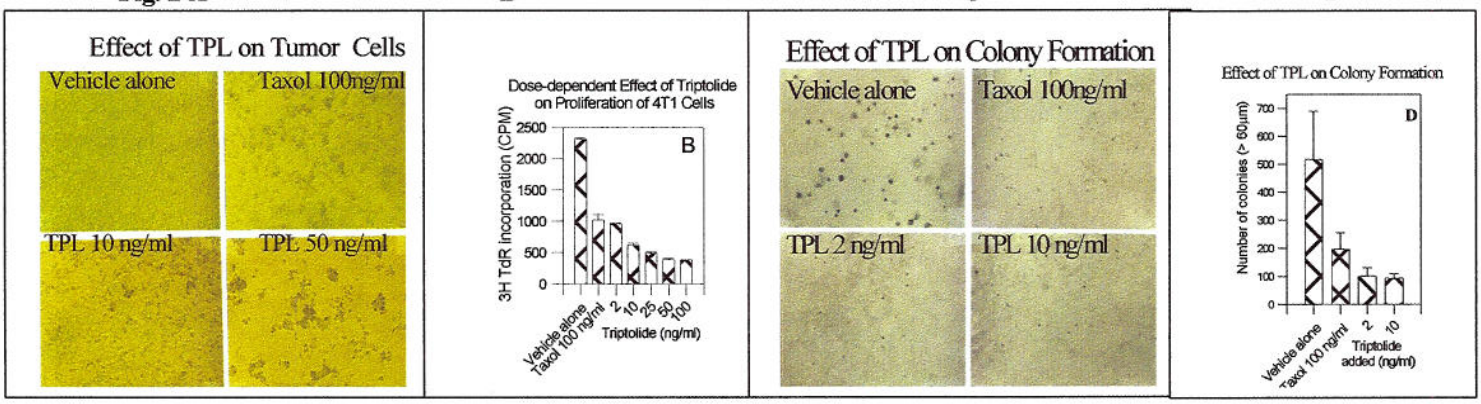
We were fortunate in being able to obtain a large amount of highly purified, crystallized TPL (**Fig. 1**) to carry out experiments with tumor cells to explore the potential of TPL as a therapeutic agent for breast cancer. The results are summarized as following:



I. Anti-Cancer Effect of TPL *in vitro*

In initial experiments, the ***in vitro*** effect of TPL on the growth of tumor cells was examined. When 4T1 breast cancer cells were treated with 10--50 ng/ml of TPL overnight, the cells appeared to be sicker (rounded and detached) than those treated with 100 ng/ml of Taxol (**Fig. 2 A**), and their **proliferation** (evaluated by ^{3}H-thymidine incorporation) was reduced in a dose-dependent manner (**Fig. 2 B**). Importantly, 2 ng/ml of TPL had an inhibitory effect equivalent to 100 ng/ml of Taxol in the treatment of both aggressive MDA-435 cells and 4T1 cells (**Fig. 2 B**), indicating that TPL is a **very potent** anti-tumor agent. Furthermore, the ability of tumor cells to **form colony** was also greatly inhibited by TPL (**Fig. 2 C**). Statistical analysis **(Fig. 2 D)** showed that while 100 ng/ml of Taxol had a 60% inhibition rate, the 2 ng/ml of TPL had a 80% inhibition rate, indicating that TPL was more potent in the suppression of tumor **colony formation**. These results were very reproducible.

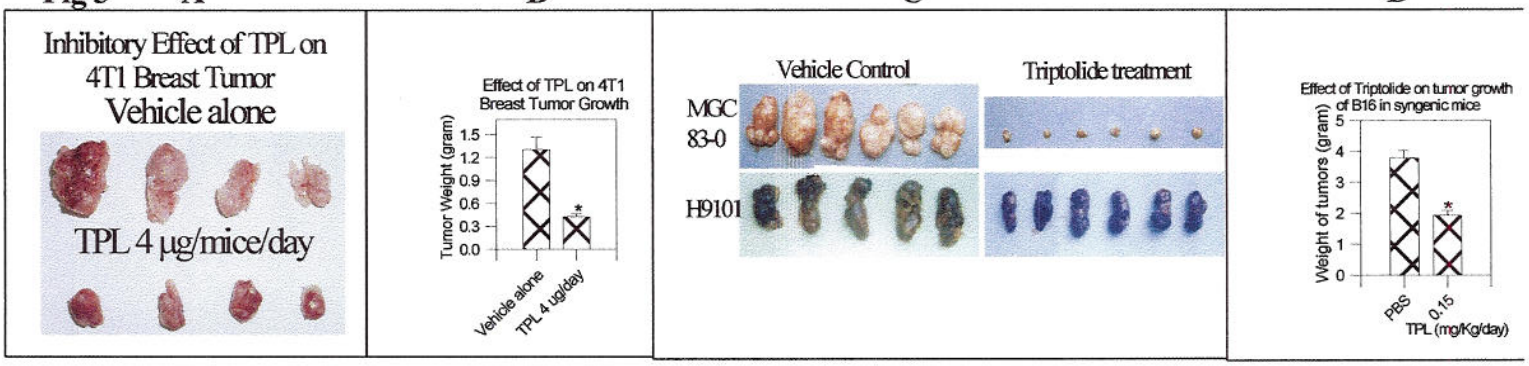
Fig. 2 A **B** **C** **D**



II. Effect of TPL on primary tumors

Similarly, the ***in vivo*** effect of TPL was very promising. The BALB/c mice that had established 4T1 **primary** breast tumors (size 0.2 cm in diameter) was i.p. injected with TPL at a dose of 4 μg/mice/day. At the end of 14 days, the tumors were harvested, photographed and weighed. **Figure 3A** shows that the tumors in TPL treated group were smaller than those in vehicle control, and this difference was statistically significant ($P<0.05$, **Fig. 3B**). To determine if TPL can inhibit the growth of **other types** of solid tumors, this drug was injected i.p. into nude mice (0.27 mg/kg/day for 21 days, equivalent to 7 μg/day) that had human xenografts of MGC80-3 gastric cancer or H9101 hepatoma of approximately 100 mm^3 in size. As shown in **Fig. 3C,** TPL caused a dramatic reduction in the size of the MGC80-3 tumors to 10% of that of controls. Similarly, TPL caused a 50% reduction in the size of H9101 tumors. In mice with B16 melanoma, the injection of TPL (4 μg/day for two weeks) resulted in a 50% reduction in the size of these very aggressive tumors (**Fig. 3D**).

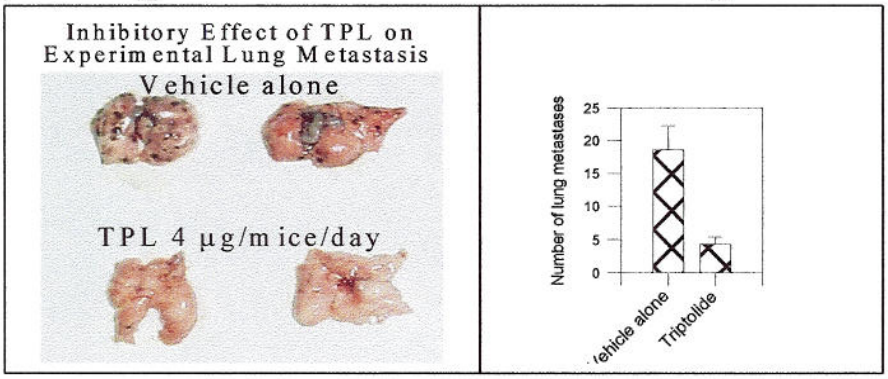
Fig 3 **A** **B** **C** **D**



III. Effect of TPL on metastatic tumor

We were more interested in the effect of TPL on **metastatic** tumor, since the metastasis is the major death cause of breast cancer patients. For this, the B16 lung metastasis model was used. Two days after injection of B16 (50,000 cells/mice) into the tail veins of C57BL/6 mice, the TPL (at 4 μg/day) were given daily injection for two weeks. **Figure 4 A** shows that the TPL treated mice had fewer **lung metastases** as compared to the control animals and this difference was statistically significant ($P<0.05$, **Fig. 4 B).**

Fig. 4 **A** **B**

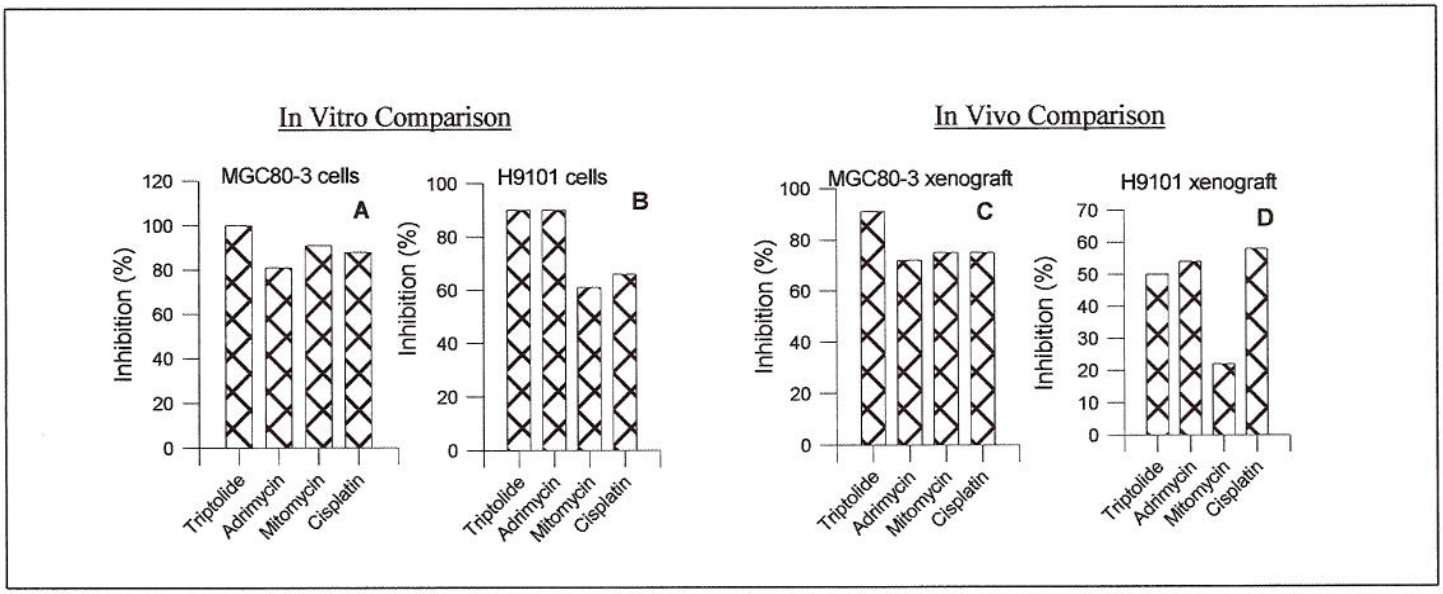


IV. Comparison anti-tumor effect of TPL with conventional chemotherapeutic drugs.

We were also eager to **compare** the inhibitory effect of TPL with **conventional** chemotherapeutic **drugs**. ***In vitro***, the cell proliferation assay was performed with MGC80-3 cells and H9101 cells, two aggressive human tumor cell lines. The tumor cells were treated with TPL at a concentration of 10 ng/ml; Adrimycin at 360 ng/ml; Mitomycin C at 2,700 ng/ml; Cisplatin at 2,490 ng/ml, respectively. These doses are commonly used *in vitro*. **Figure 5A and B** shows that TPL had a 90% inhibition rate on these two malignant cell lines, which was compatible with three conventional chemotherapy drugs tested. In the case of H9101 cells, the inhibitory effect of PTL was even better than Mitomycin (90% vs. 60%) and Cisplatin (90% vs. 68%).

Importantly, these *in vitro* results were translatable in animal models. When the nude mice that had xenografts of MGC80-3 or H9101 tumors (100 mm^3 in size) were injected with TPL at 0.27 mg/kg daily; Adrimycin at 1.2 mg/kg weekly; Mitomycin at 1.7 mg/kg weekly and Cisplatin at 7 mg/kg weekly, respectively for three weeks. . These doses are commonly used in tumor animal models. The results were similar to those obtained with the *in vitro* experiments, in that TPL demonstrated an inhibitory effect (**Fig. 5 C and D**) on both tumor models that was comparable to that of Adrimycin, Cisplatin and Mitomycin. These results further support that TPL has a great potential as a new anti-tumor agent.

Fig. 5 Comparison of TPL with other chemo-drugs



V. The efforts in changes of formulation to improve the therapeutic window

In all our *in vivo* studies, when treated with TPL at 0.15 mg/kg/day (equivalent to 4 μg/day) for two weeks, all of the mice well tolerated with TPL without obvious sickness signs or the loss of weight at the end of experiment (data not shown). A few BALB/c mice (syngenic mouse for 4T1 breast cancer) showed some signs of sickness when were treated with 0.27 mg/kg/day for two week, indicating that an optimal dose should be carefully worked out before going to any clinical trials.

Dr. Reutrakul and coworkers have reported that a modest anti-tumor effect was observed with an i.p. dose of 25 μg/mice every other day (3). The dose different between this study and our study may due to the purity of TPL. However, the higher dose of 50 μg/mouse three times weekly was lethal (3). It seems that the therapeutic window is relative small.

TPL possesses a small molecule weight (MW 360 Dalton), therefore, it is easy to randomly diffuse out of vessels and accumulated in major organs, such as liver and kidney, and cause the adverse side-effect (personal communication with physicians carrying clinical trial in China).

In our hand, the therapeutic window (LD_{50}/ED_{50}) of TPL is about 4. We believe that before we go to any clinical trial with TPL in USA, it would be better off if we can develop TPL into a new form that targets on tumor with a relatively high specificity.

When we searched for the now formulation, we found that **liposome** was used as a carrier for Doxorubicin. The liposomal Doxorubicin (DOXIL) at a dose of **25** mg/M^2 exerts an anti-cancer effect as good as its parental Doxorubicin at **75** mg/M^2 with a much low toxicity (13-19). This formulation has been **approved by FDA** and is widely used in cancer treatment. The rationale of this liposomal formula is based on the tumor vessel biology that the **tumor vessels are far more loosen and leaky than normal vessels** (22-25). While Doxorubicin (MW of 543.34 Dalton) can diffuse to **heart** tissue easily, the liposome enraptured Doxorubicin with a diameter about 100 nM can not diffuse out into normal tissue easily, but can get into tumor site via their leaky vessels (13-21).

VI. Preparation of RGD/NGR-PA-TPL-liposome

Trigged by liposomal Doxorubicin (DOXIL), we decided to make RGD/NGR-PA-TPL-liposome to see if this new form is more potent and safer than TPL alone.

RGD/NGR-PA-TPL-liposome has two advantages: First, it increases the size, which will preferentially leaks out in the tumor site. Secondly, it specifically targets on tumor endothelial cells via $\alpha v\beta_3$ integrin and CD13.

The **RGD** is a ligand of $\alpha v\beta_3$ and $\alpha v\beta_5$ integrins that are expressed on both tumor cells and tumor endothelial cells (26-29). While the RGD alone has no effect on the tumor cells (30), the RGD linked peptide can better target tumors via the integrins on their surface (27, 28). The internalization of RGD tagged TPL through the $\alpha v\beta_3$ and $\alpha v\beta_5$ integrin-mediated pathway may facilitate a specific killing of tumor endothelial cells (27, 28).

The **NGR** peptide motif is an aminopeptidase N (CD13) ligand that targets on the angiogenic blood vessels. Immunohistochemical staining showed that CD13 expression is up-regulated in human tumors, but is not detected in blood vessels of various other normal tissues stained under the same conditions (32). The **NGR** peptide motif has been used to deliver cytotoxic agents, such as pro-apoptotic peptides and tumor necrosis factor-alpha (TNF), to tumor vasculature. (27, 28, 31). The drug coupled to an NGR peptide has more potent anti-tumor effect than the free parental drug (28).

Our idea was to utilize the peptide motifs of RGD and NGR to specifically deliver of TPL to tumor vessels. The RGD motif was linked to NGR motif via glycine link domain. This design would allow the endothelial cells to bind to either RGD or NGR depending on the number and the affinity of $\alpha v\beta_3$ and CD13 on their surface, which would increase the targeting chance.

In addition, our idea was combined with another idea that was triggered by recent report in *Science* from Dr. Cheresh group, in which the targeted nanoparticle (NP) was used (33). The $\alpha v\beta_3$ ligand was covalently linked to cationic lipid that formed nanoparticle and enraptured with mutant Raf gene. This targeted gene delivery to the tumor neovasculature resulted in a sustained regression of established primary and metastatic tumor (33). We believe that if we **conjugate TPL to RGD and NGR peptides** which have a **lipid tail** that **can anchor on the bio-membrane of** liposome (Lipo), then this special targeting liposome has not only a **big size** that can pass **leaky** tumor vessel, but also a **specifical target** on the tumor neovasculature via interaction with $\alpha v\beta_3$ integrin and CD13.

The form of liposome was PEG-DSPE (1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Carboxy(Polyethylene Glycol)2000, Avanti Polar Lipids Inc.), since it represents the best backbone of liposome. The PEG confers DSPE liposomes with a brush-like coating, which will prevent the DSPE liposomes from nonspecific opsonization by plasma proteins. The PEG can also interrupt the recognition and clearance by the macrophages and other elements of the reticuloendothelial system. Therefore, it should **increase the half-life** of liposomes in circulation and facilitate the **accumulation of liposomes in tumors** (34-37).

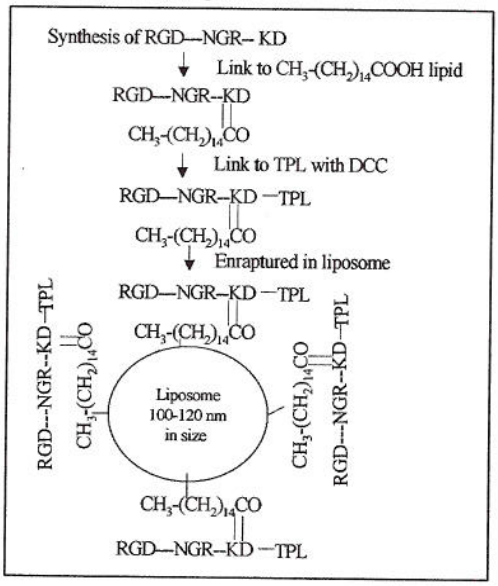
Our detailed preparation of **RGD/NGR-PA-TPL-Lipo** is described as following:

First, the RGD/NGR peptide was synthesized with a lysine (R) and aspartic acid (D) tail, which allow to conjugate palmitic acid {CH_3-$(CH_2)_{14}$COOH} lipid and TPL later on.

Secondly, the palmitic acid (PA) lipid was linked to R when the synthesis of RGD/NGR peptide targeting motif was finished to form RGD/NGR-PA.

Thirdly, the RGD/NGR-PA was linked to TPL with N,N'-Dicyclohexylcarbodiimide (DCC) at the site of D to form RGD/NGR-PA-TPL.

The HPLC was used to purify the RGD/NGR-PA-TPL and the analysis indicated the purity was 98%. The NMR data showed an expected molecule was synthesized.



Finally, the RGD/NGR-PA-TPL was mixed with PEG-liposome to form targeting RGD/NGR-PA-TPL-lipo in a size of 100-120 nm. The PA lipid was merged in the biolipid layer and the RGD/NGR head had 50% chance to expose on the surface of liposome.

VII. Testing effect of RGD/NGR-PA-TPL-liposome

After obtained 300 mg of RGD/NGR-PA-TPL-liposome, we were able to test its effect both *in vitro* and *in vivo*.

A) Effect of RGD/NGR-PA-TPL-liposome *in vitro*: The equal amount of 4T1, MDA231 breast cancer cells or HUVEC (human umbilical cord veil endothelial cells), respectively, were seeded in 96 well plates (triplicate per group) and then treated with different concentrations (0, 1, 2, 5, 10 ng/ml) of TPL or RGD/NGR-PA-TPL-lipo. At the different time points (24, 48 and 72 hr post-treatment), the cell proliferation in each treatment group was determined with 3H-TdR incorporation as described above. The results showed that there was no much difference of the effect on inhibition of cancer cell proliferation between the TPL and RGD/NGR-PA-TPL-lipo.

B) Effect of RGD/NGR-PA-TPL-liposome *in vivo*: To test the *in vivo* effect on metastatic breast tumors, as proposed, the 4T1 mouse breast cancer cells (2 X 10^5) were i.v. injected into BALB/c mice. The tumors were allowed to establish for three to five days, then the mice will randomly divided into groups and treated with different doses (0, 0.05, 0.1, 0.2, 0.3, 0.5, 1, 2 and 4 mg/kg) of TPL or its derivatives. The groups were: **1)** vehicle alone; **2)** TPL alone; **3)** TPL-liposome; and **4)** RGD/NGR-PA-TPL-lipo (test group).

The treatment was delivered via i.v. injection and the schedule was twice a week for 4-5 weeks.

The breathing rate of mice with 4T1 lung metastases was carefully observed every day.

By the end of week 3, 6 injections of above agents were executed. However, we noted that the tail veins that received RGD/NGR-PA-TPL-lipo injections were blocked and the nearby arteries were affected the as evidenced by the whole tail became "black and dry". This deleterious side effect did not occur in three other treatment groups, strongly suggesting that the **RGD/NGR does bind to the vessel endothelium and enhances the cytotoxicity effect of TPL on local vessels.**

While we were very happy with this fact and closed to prove that our idea was correct, we were shocked by the 7th injections that were carried out in week 4. Immediately after i.v. injection, the mice had a ruffled hair, up-set and a fast breathing, and gradually, they had lethargy, less movement, and breathing difficult. Within 30 minutes, all the mice receiving 7th injections at week 4 dead. This dismay result strongly indicated there was an **IgE type super-acute immune reaction**. The allergy source should be the RGD/NGR peptide, since the rest of other group did not have a similar symptom and other components in the molecule were unlikely to sever as an antigen.

At this time point, we decided not to further pursue the testing of RGD/NGR-PA-TPL-lipo in other models (such as MDA231 primary breast cancer model in nude mice), since the side effect of lethal allergy was overwhelming and any further use of this molecule was unwise.

VIII. Preparation and test of HA (hyaluronan)-TPL-liposome both *in vitro* and *in vivo*

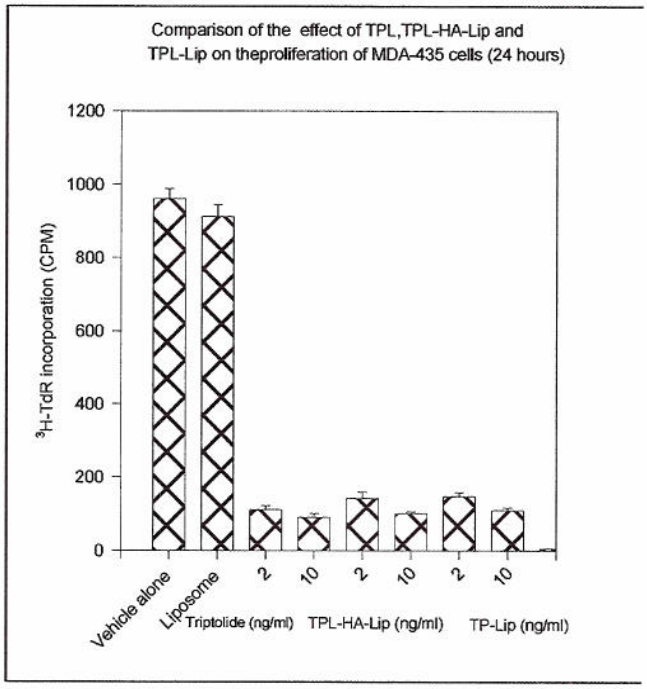
The lesson from using RGD/NGR-PA-TPL-lipo leads us to avoid the use of any human peptide or protein as a targeting molecule in mouse model. We decided to use hyaluronan (HA), a polysaccharide that is universal in all spices without any antigenicity. HA is chosen as a new carrier based on that it is the ligand for CD44, which is highly expressed in most breast cancer cells, including the cancer stem cells.

Using a chemical reaction, we were able to conjugate HA to TPL and to prepare its liposome form (TPL-HA-Lipo). The control was parental TPL and TPL- Lipo.

The *in vitro* test showed that the chemical reaction did not destroy the bioactivity of TPL as evidenced by that the HA-TPL-lipo had a similar activity as parental TPL (**Fig 6**). However, there was no significant difference of the inhibitory effect on the proliferation of MDA435 tumor cells among the parental TPL, TPL-Lipo (control) and TPL-HA-Lipo (test group for the enhancement effect of carrier).

In this in vitro system, the effect on vessel endothelium that also expressed a high level of CD44 receptor was not tested.

Fig 6. Effect of TPL, TPL- Lipo (control) and TPL-HA-Lipo in vitro

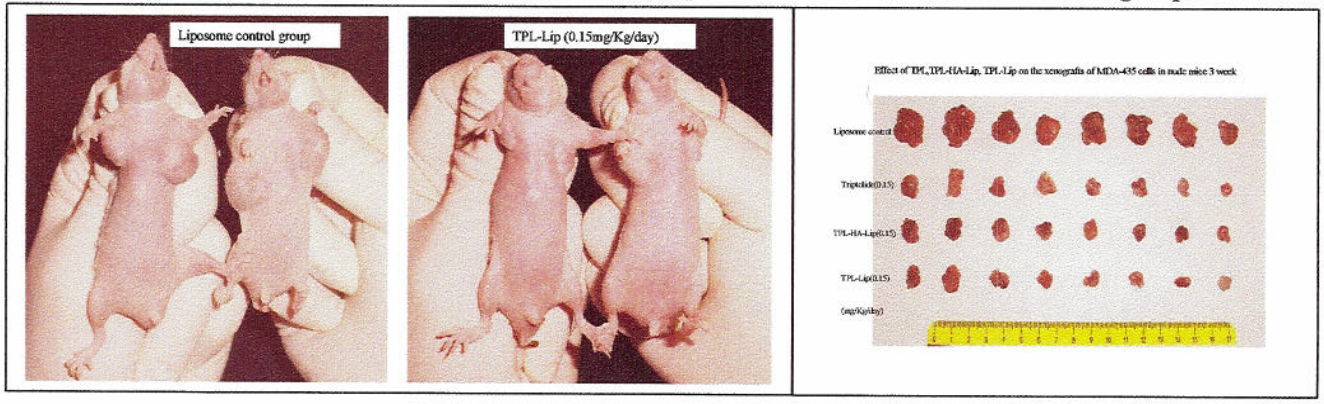


The in vivo tumor bearing mouse model would allow us to test the overall effect of TPL-HA-Lipo on both cancer cells and their vessel endothelium, affecting the growth of tumor.

The nude mice bearing with 60 mm^3 of human MDA 435 tumor were randomly divided into four groups and treated with **1)** liposome alone as vehicle control; **2)** TPL alone as parental agent; **3)** TPL-Lipo as no targeting carrier; and **4)** TPL-HA-Lipo as test group for targeting carrier. The TPL in different group was used equally, i.e. 0.15 mg/kg. The agents were delivered via i.v. every other day for three weeks. The tumor sizes were measured twice per week. At the end of experiment, the mice were weighted for the evaluation of toxicity, and the tumors were pictured and weighted for the anti-tumor effect of agents.

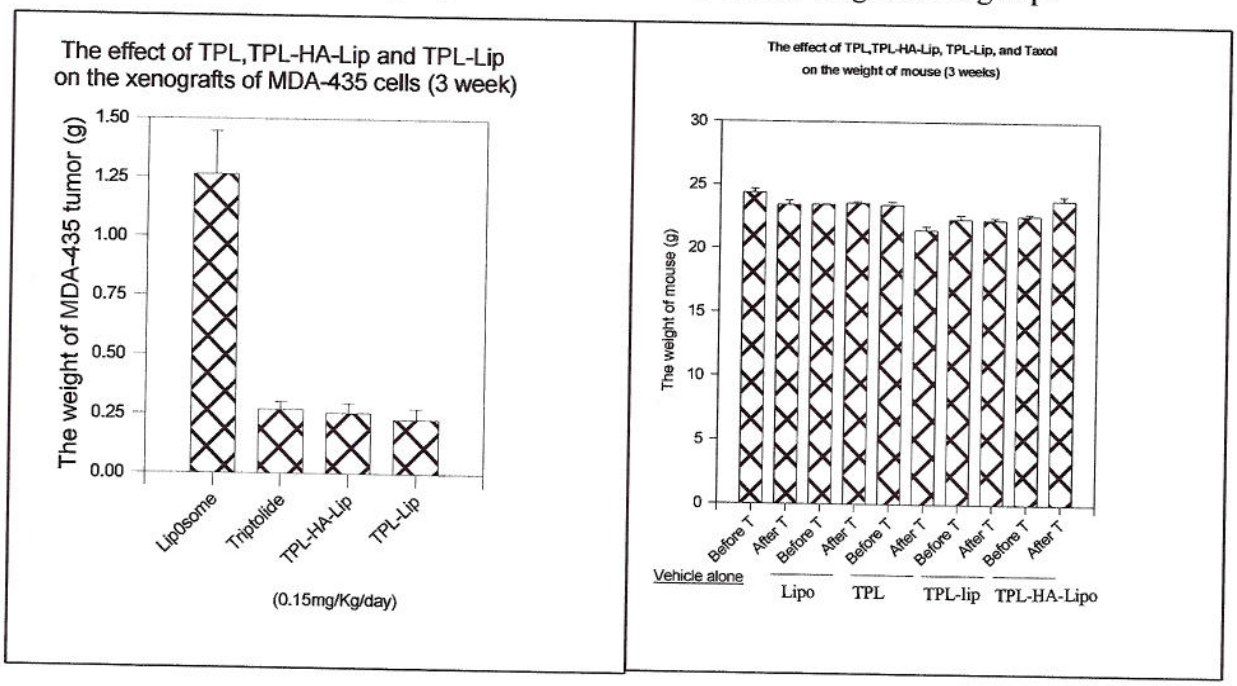
The result showed that the liposome alone group had a fast growing big tumor (**Fig 7A**) while all other groups with TPL (equal amount of TPL at 0.15 mg/kg) had small tumors (**Fig 7B-C**).

Fig 7A Liposome alone group **B** TPL-Lipo group **C** Tumors from all groups



The results of tumor weight showed that the liposome alone group had a mean tumor weight of 1.25 gram while mean tumor weight in all other TPL groups had only 1/5 of that (about 0.25 gram, **Fig 8A**). There was no difference of therapeutic effect of TPL among the different formulations of TPL, although there was not side effect of all formulated agents as evidenced by the mice body weight did not change much before and after administration of agents for three weeks (**Fig 8B**).

Fig 8A Tumor weights of all groups B Tumor weights of all groups



These data suggest that at the current format and animal model, the HA carrier does not help in targeting of both tumor cells and tumor endothelium, and the TPL alone exerts the anti-tumor effect as well as its liposome form and HA conjugated form. At this point, the only explanation is that the TPL is a hydrophobic molecule, which tends to leak out of liposome and existing as an original form. The conjugation with HA is likely to make the molecule too big to freely penetrate into cells, and the requirement of endocytosis for entry of cells may reduce the efficacy of TPL-HA-Lipo.

Taken together, TPL is a potent anti-tumor agent. Although the targeting nanoparticles formulation of TPL (RGD/NGR-PA-TPL-liposome) sounds good and wealth to try out, it causes a devastating allergy side effect due to the antigenicity of RGD/NGR in mouse model. The HA as carrier is reasonable for targeting CD44, a cancer stem cell marker, however, the big size of HA is likely to prevent the TPL free-movement inside the tumor cells. The hydrophobic property of TPL makes it not suitable to formulate as a liposome form.

While how to enhance the efficacy of TPL is still a challenge, the use of TPL itself as anti-cancer and anti-inflammation agent has been widely accepted. We will continue our study alone this line.

Key Research Accomplishments

- RGD/NGR-PA-TPL-liposome was obtained.
- RGD/NGR enhanced the targeting effect on vessel.
- This study defined that human RGD/NGR could act as foreign antigen to mouse, perhaps for human too. Therefore, it is not a good carrier to anti-tumor toxic agent.
- TPL-HA-liposome was obtained.
- The TPL, TPL-Lipo and TPL-HA-Lipo had a similar effect both *in vitro* and *in vivo*.

Reportable Outcomes

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Conclusions:

1) When peptide is used as a carrier for targeting cell surface molecules, it is critical to consider if the peptide can be a foreign antigen to the host and cause a dangerous immune reaction that can lead to an overwhelming side effect.

2) TPL itself is potent enough to kill cancer cells without further modification.

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